Glyoxalase-1 overexpression in bone marrow cells reverses defective neovascularization in STZ-induced diabetic mice

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Aims
Methylglyoxal (MG) accumulates in diabetes and impairs neovascularization. This study assessed whether overexpressing the MG-metabolizing enzyme glyoxalase-1 (GLO1) in only bone marrow cells (BMCs) could restore neovascularization in ischaemic tissue of streptozotocin-induced diabetic mice.

Methods and results
After 24 h of hyperglycaemic and hypoxic culture, BMCs from GLO1 overexpressing and wild-type (WT) diabetic mice were compared for migratory potential, viability, and mRNA expression of anti-apoptotic genes (Bcl-2 and Bcl-XL). In vivo, BMCs from enhanced green fluorescent protein (eGFP) mice that overexpress GLO1 were used to reconstitute the BM of diabetic mice (GLO1-diabetics). Diabetic and non-diabetic recipients of WT GFP + BM served as controls (WT-diabetics and non-diabetics, respectively). Following hindlimb ischaemia, the mobilization of BMCs was measured by flow cytometry. In hindlimbs, the presence of BM-derived angiogenic (GFP + CXCR4 + ) and endothelial (GFP + vWF + ) cells and also arteriole density were determined by immunohistochemistry. Hindlimb perfusion was measured using laser Doppler. GLO1-BMCs had superior migratory potential, increased viability, and greater Bcl-2 and Bcl-XL expression, compared with WT BMCs. In vivo, the mobilization of pro-angiogenic BMCs (CXCR4 +, c-kit +, and Flk +) was enhanced post-ischaemia in GLO1-diabetics compared to WT-diabetics. A greater number of GFP + CXCR4 + and GFP + vWF + BMCs incorporated into the hindlimb tissue of GLO1-diabetics and non-diabetics than in WT-diabetics. Arteriole and capillary density and perfusion were also greater in GLO1-diabetics and non-diabetics.

Conclusion
This study demonstrates that protection from MG uniquely in BM is sufficient to restore BMC function and neovascularization of ischaemic tissue in diabetes and identifies GLO1 as a potential therapeutic target.

Keywords
Neovascularization • Type 1 diabetes • Methylglyoxal • Bone marrow cells

1. Introduction
Following ischaemia, neovascularization occurs to restore the supply of oxygen and nutrients. Blood vessel regeneration involves signalling from ischaemic tissue and mobilization of both local pro-angiogenic cells and bone marrow-derived circulating angiogenic cells (BM-CACs). BM-CACs are a heterogeneous population consisting mostly of myeloid haematopoietic cells, with a small fraction of true endothelial precursors. Although phenotypic and functional characterization of these cells has been controversial, subsets of these cells can contribute to vascular repair through direct participation in the formation of new blood vessels, but primarily through the secretion of pro-angiogenic cytokines and the recruitment and regulation of local angiogenic cells. Decreased vascularity and defective ischaemia-induced neovascularization are major contributors to cardiovascular complications in diabetes. Hyperglycaemia impairs neovascularization through inactivation of the hypoxia-inducible factor-1α (HIF-1α) that regulates angiogenic factors, including vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1). Furthermore, hyperglycaemia attenuates the tissue’s signalling capacity to recruit BM-CACs and,
through the generation of toxic metabolic glucose by-products, creates a hostile environment for recruited cells. Additionally, bone marrow cells (BMCs) themselves are functionally deficient in diabetes, characterized by a poor ability to mobilize in response to hypoxia and to promote blood vessel growth.

Hyperglycaemia and the related formation of advanced glycation end-products (AGEs) have been associated with the pathogenesis of diabetic vasculopathy. AGEs are formed by non-enzymatic protein glycation, i.e., the addition of sugars and toxic aldehydes, such as methylglyoxal (MG) and glyoxal. Through interaction with the receptor for AGE, AGEs increase inflammation and oxidative stress by the formation of reactive oxygen species (ROS). MG is the major glycating agent in diabetes, and MG-derived hydroimidazolones are the predominant AGEs in tissues of diabetic patients. Detoxification of MG is done primarily by the glyoxalase system: glyoxalase-1 and -2 (GLO1 and GLO2); in the presence of reduced glutathione, MG is converted into S-lactoylglutathione. Overexpression of GLO1 reduces AGE formation and ROS. Decreasing intracellular ROS by increasing GLO1 expression can rescue angiogenic cells from hyperglycaemia-induced functional defects in vitro, through reversal of the MG-mediated HIF-1α modification. Transgenic rats over-expressing human GLO1 (hGLO1) showed reduced retinal, neuroglial, and vascular pathology following treatment with streptozotocin (STZ) and were resistant to renal ischaemia–reperfusion injury.

Since BM-CACs are one of the major contributors to vascular repair, the objective of this study was to look specifically at the role of dicarbonyl stress (increased oxidation of glucose and inadequate detoxification) caused by MG in the BM. We examined whether over-expression of hGLO1 in the BM could help restore lost BM mobilization and BM-CAC function and reverse the defective ischaemia-induced neovascularization in an STZ-induced mouse model of Type 1 diabetes.

2. Methods

2.1 Transgenic hGLO1 mice

All animal procedures were performed with the approval of the University of Ottawa Animal Care Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The cDNA encoding hGLO1 with an amino terminal c-myc epitope tag was cloned into the Not1-digested PEP8 plasmid, so that the hGLO1 insert was under the control of the murine pre-proendothelin promoter. The PEP8 plasmid was kindly provided by Dr William Sessa (Boyer Center for Molecular Medicine, Yale University) with the permission of Dr Mitsuhiro Yokoyama (Kobe University School of Medicine). The ~13 kb Spel-linearized PEP8hGLO1 was micro-injected into the pronuclei of fertilized C57BL/6 mouse eggs that were then transferred to the oviducts of pseudo-pregnant foster mothers. Transgenic founders were identified by PCR screening. All experiments used mice hemizygous for the hGLO1 transgene from a single line (hGLO1 mice) or their non-transgenic littermates.

2.2 Diabetes induction

C57BL/6 mice received an intraperitoneal injection of STZ (50 mg/kg) in 0.05 M sodium citrate or an equal volume of citrate buffer (control non-diabetic mice) for 5 consecutive days. Blood glucose measurements of fasting animals were taken 8–10 days after the first STZ injection and following fasting sacrifice. Mice with glucose levels >15 mmol were considered hyperglycaemic/diabetic; those below this level were excluded. Mice were fed the Teklad Global 2019 Extruded Rodent Diet (Harlan, Mississauga, Canada).

2.3 Bone marrow extraction for in vitro studies

Three groups of mice were used for the in vitro study: STZ-treated hGLO1 transgenic mice (GLO1-diabetic) and their wild-type (WT) littermates divided into STZ-treated (WT-diabetic) and citrate buffer-treated (WT) groups. Mice were anaesthetized under 3% isoflurane, and euthanasia was performed by an overdose of sodium pentobarbital. Bone marrow was harvested from femurs and tibias using a 27-gauge needle and phosphate buffered saline. The BMCs were collected 4 weeks after confirmation of hyperglycaemia, separated by density gradient centrifugation (Histopaque 1119; Sigma, Oakville, Canada), and counted (Vicell™ Analyzer; Beckman Coulter, Mississauga, Canada). As a measure of oxidative stress, BMC lysates were prepared for analysis using the OxiSelect protein carbonyl ELISA kit, following the manufacturer’s protocol (Cell Biolabs, San Diego, USA). Cells were cultured on fibronectin-coated plates in endothelial basal medium (EBM; Clonetics, Mississauga, Canada) with gentamycin, lacking growth factors and serum, but containing either 30 mmol glucose for BMCs from GLO1-diabetic and WT-diabetic mice or 5 mmol glucose for BMCs from non-diabetic WT mice.

2.4 In vitro viability assay

BMCs (n = 3 per group) were cultured for 24 h (as described above) under hypoxic conditions (1% O2 and 5% CO2), and stained using the LIVE/DEAD® Viability Assay Kit (Invitrogen, Burlington, Canada). Cells were imaged using an Olympus BX60 microscope with the Spot Basic software (four images/sample, ×10 magnification). Viability was calculated per field-of-view (FOV) as: viable cells/total cells.

2.5 Chemotaxis migration assay

Migration was assessed using a horizontal fibronectin-coated μ-Slide Chemotaxis assay (Ibidi, Verona, USA). Briefly, 3 × 10^4 BMCs were loaded into the observation area and allowed to adhere for 2 h at 37°C and 5% CO2 followed by two rinses with serum/growth factor-free EBM. The adjacent reservoir was filled with serum/growth factor-free EBM and one of the following: (i) SDF-1 (50 ng/mL), (ii) VEGF (50 ng/mL), or (iii) blood serum collected from diabetic mice sacrificed 3 or 7 days after hindlimb artery ligation surgery to induce ischaemia (as described below). The media contained 30 mmol glucose for diabetic BMCs, or 5 mmol glucose for non-diabetic BMCs, as described above. Slides were incubated for 18 h at 37°C, and the number of cells that migrated a distance of ≥9 mm towards the chemotactic attractant was visualized and quantified using microscopy.

2.6 Animal model and BM transplant

Mice hemizygous for the hGLO1 transgene were crossed with enhanced green fluorescent protein transgenic mice [eGFP mice; C57BL/6-Tg(CAG-EFPl-EGFP)1Oibs/J; Jackson Laboratories, Bar Harbor, USA], and their progeny were identified by PCR genotyping and used as BM donors (eGFP or hGLO1/eGFP BMCs).

BM transplantation (BMTx) was performed as previously described. Briefly, recipient C57BL/6 male mice (8- to 10-week-old; Jackson Laboratories) maintained on antibiotic-containing drinking water (NovoTrimel; 1.5 mL/100 mL of water) for 1 week were irradiated with two equal 450 rad doses, 3 h apart, using a caesium source. Donor BMCs (eGFP+ or hGLO1+/eGFP+) were freshly isolated as described above, but without gradient separation. BMCs (7 × 10^6 cells/animal) were injected into the tail vein, and 4 weeks later the mice received intraperitoneal injections of STZ (WT-diabetic and GLO1-diabetic groups) or an equal volume of citrate buffer (non-diabetic mice). Four weeks after confirmation of hyperglycaemia, hindlimbs ischaemia was induced by ligation of the left proximal femoral artery, under 3% isoflurane (inhaled), as previously described. Pain was managed pre-operatively and 1 and 2 days after surgery by buprenorphine (subcutaneous).
2.7 Glyoxalase activity

GLO1 activity was assayed by measuring the rate of formation of S-(α-lactoylglutathione from hemi-thioacetal, as described. Briefly, the assay mixture consisted of 7.9 mM Mg, 1 mM glutathione, 14.6 mM MgSO₄, and 182 mM imidazole HCl pH 7.0. Following equilibration, the reaction was initiated by the addition of BMC lysate (20 μg). GLO1 activity is reported as millimolar of S-(α-lactoylglutathione formed/min/mg of lysate protein (concentration determined by the Lowry protein assay).

2.8 Laser Doppler analysis

Under 3% isoflurane (inhaled), hindlimb blood perfusion was measured using laser Doppler (moorLD2; Moor Instruments, Axminster, UK), pre-operatively, immediately following surgery, and at 7 and 14 days post-ligation, as described. The results are expressed as the ratio of ischaemic to non-ischaemic hindlimb blood flow.

2.9 Flow cytometry

Flow cytometry was performed on circulating GFP+ cells collected by saphenous vein bleeds pre-operatively and at Days 1, 4, 7, and 14 post-surgery, as described previously. Briefly, the mononuclear cell fraction was labelled with antibodies against the following antigens: c-kit (Southern Biotech, Birmingham, USA), CXCR4 (BD Biosciences, Mississauga, Canada), and flk-1 (mouse vascular endothelial growth factor receptor-2; ebioscience, San Diego, USA), and analysed with a FACSAria flow cytometer (BD Biosciences). The fold-change in the percentage of positive cells for early (Days 1–4) and late (Days 7–14) response times was calculated relative to baseline, as described previously. For BMC characterization pre-BMTx, the mononuclear cell fraction was labelled with c-kit, CXCR4, flk-1 (same suppliers as above), and CD34 antibodies (BioLegend, San Diego, USA). The FACSAria flow cytometer was also used for the sorting of CXCR4+/CD34+ and CXCR4−/CD34− cells from the BM in order to measure hGlo1 mRNA expression in these BMC populations.

2.10 Immunohistochemistry

Two weeks after ligation surgery, mice were sacrificed as described above, and hindlimb muscle tissue distal to the ligation site (medial thigh muscle) was frozen in optimum cutting temperature compound, sectioned, and fixed with methanol. Sections (10 μm) were stained with an α-smooth muscle actin antibody for arterioles, von Willebrand factor (vWF) or CD31 antibodies for endothelial cells, CXCR4 antibody for angiogenic cells, and GFP antibody for recruited BMCs (all antibodies from Abcam). Protein was transferred onto polyvinylidene fluoride membranes (Immobilon-FL, Millipore, Toronto, Canada), blocked in 5% non-fat dry milk in TBS—Tween 20 buffer for 1 h before probing with primary antibodies. Incubation with anti-cmyc 9E10 (1 : 1000; ATCC, Manassas, USA), anti-GFP, and anti-eNOS (both 1 : 100; Abcam, Cambridge, UK) antibodies was performed overnight at 4°C. Secondary antibodies were from Cell Signaling Technology (Whitby, Canada). Signal detection was performed with the SuperSignal West Pico chemiluminescence kit (Pierce, Ottawa, Canada). Protein concentration was determined by the BCA assay (Thermo Scientific, Ottawa, Canada). Western blot band intensity was determined using the ImageJ software.

2.11 Cytokine antibody arrays

Relative cytokine levels were analysed in hindlimb tissue lysates (100 μg) or blood serum (50 μL) from sacrificed animals (n = 4–6 per treatment group) using RayBio Mouse Cytokine Antibody Array Kits (cat# AAM-ANG-G1-B; Raybiotech, Norcross, USA), according to the manufacturer’s protocol.

2.12 RNA extraction, cDNA synthesis, and quantitative PCR

Total RNA was extracted from muscle or cultured BMCs using Trizol reagent (Invitrogen), following the manufacturer’s instructions. First-strand cDNA was synthesized from RNA (2 μg) using GoScript reverse transcriptase (Promega, Madison, USA) and random hexamer primers (IDT, Toronto, Canada). mRNA levels were assessed by real-time quantitative polymerase chain reaction (RT-qPCR) using BRYT Green GoTaq qPCR Master Mix (Promega) and a LightCycler 480 Real-Time PCR system (Roche, Mississauga, Canada). Primer pairs (see Supplementary material online, Table S1) were designed using the DNAMAN software (Lynnon Biosoft, Pointe-Claire, Canada) and primer3 (v0.4.0). Relative changes in mRNA expression of target genes were determined using the ΔΔCt method, expressed as levels relative to the combined average values of 18S and Gapdh.

2.13 Bone marrow and tissue western blot analysis

BMCs and hindlimb tissue were lysed for protein extraction using RIPA buffer (with protease inhibitor; Roche). Equal amounts of protein (150 μg for BMCs, 40 μg for hindlimb tissue) were loaded into 10% Precise Protein Gels (Fisher, Ottawa, Canada) and resolved by SDS—PAGE. For detection of endothelial nitric oxide synthase (eNOS) dimers, polyacrylamide electrophoresis was performed at low temperature (4°C), as previously described. Protein was transferred onto polyvinylidene fluoride membranes (Immobilon-FL, Millipore, Toronto, Canada), blocked in 5% non-fat dry milk in TBS—Tweek 20 buffer for 1 h before probing with primary antibodies. Incubation with anti-cmyc 9E10 (1 : 1000; ATCC, Manassas, USA), anti-GFP, and anti-eNOS (both 1 : 100; Abcam, Cambridge, UK) antibodies was performed overnight at 4°C. Secondary antibodies were from Cell Signaling Technology (Whitby, Canada). Signal detection was performed with the SuperSignal West Pico chemiluminescence kit (Pierce, Ottawa, Canada). Protein concentration was determined by the BCA assay (Thermo Scientific, Ottawa, Canada). Western blot band intensity was determined using the ImageJ software.

2.14 Statistical analysis

All results are expressed as the mean ± SEM. Comparisons of continuous data between groups were performed with a one-way analysis of variance, and that between individual groups were performed with a two-tailed Student’s t-test using the SigmaStat software. P ≤ 0.05 were considered statistically significant.

3. Results

3.1 Characterization of hGlo1 transgenic mice

The immunoreactive cmyc-hGlo1 was detected by western blotting in all tissues tested in hGlo1+/−/+ mice (heart, aorta, kidney, eye, liver, and brain—data not shown). Despite the reported endothelial-specific expression of this promoter, extracts of isolated endothelial, smooth muscle cells, and BMD-derived macrophages from hGlo1+/− mice had approximately five-fold greater GLO1 activity than those from non-transgenic littermates (see Supplementary material online, Table S2). Immunohistochemistry of mouse aortas also indicated that transgene expression was not restricted to the endothelium (not shown). The hGlo1 mRNA expression was confirmed through RT-qPCR in CXCR4+/CD34+ and CXCR4−/CD34− BMCs of transgenic mice. It is, therefore, clear that expression of the hGlo1 transgene is not restricted to endothelial cells.

3.2 GLO1 overexpression improves in vitro viability and reduces oxidative stress in BMCs

After 24 h in apoptosis-inducing conditions (serum deprivation, hypoxia, and high glucose), the survival of BMCs from GLO1-diabetic mice (75.4 ± 3.3%) was equivalent to that of non-diabetic WT BMCs (75.2 ± 3.9%; P = 1.0); whereas BMCs from WT-diabetic mice had...
significantly reduced survival (57.7 ± 2.9%; \(P \leq 0.024\); Figure 1A). Consistent with the increase in survival, mRNA expression of two anti-apoptotic factors, Bcl-2 and Bcl-XL, was increased in BMCs from GLO1-diabetic mice (5.5 ± 1.1 and 4.7 ± 2.3, respectively) compared with those from WT-diabetic mice (1.3 ± 0.8 and 1.8 ± 0.5, respectively; \(P \leq 0.0014\); Figure 1B). As a measure of oxidative stress, protein carbonyls in BMCs of GLO1-diabetic mice were reduced by 13-fold compared with non-diabetic WT BMCs, and also by 25-fold compared with WT-diabetic BMCs (\(P \leq 0.049\); Supplementary material online, Figure S1).

### 3.3 GLO1 overexpression maintains migratory potential of diabetic BMCs

Testing the ability of BMCs to undergo directional migration along a gradient of VEGF or SDF showed that hGlo1 overexpression increases the number of migrating cells. The percentage of cells responding (by migrating \(\geq 9\) mm) to either VEGF or SDF-1 chemo-attractant over an 18 h period was significantly lower for BMCs from WT-diabetic mice (0.3-fold, \(P \leq 0.018\); Figure 1C and D) compared with control-BMCs, while the GLO1-BMC response was similar to that of the control-BMCs.
Since pro-angiogenic/BM mobilization cytokine levels are reduced in the circulation of diabetic animals, serum that was collected from diabetic mice 3 and 7 days after undergoing hindlimb ligation was used as a chemo-attractant to examine the BMC migratory response towards physiological signals generated by diabetic ischaemic tissue. Day 3 serum attracted approximately two times more GLO1-diabetic BMCs than WT-diabetic BMCs ($P = 0.02$; Figure 1E), while Day 7 serum attracted 3.3-fold more BMCs from GLO1-diabetic mice than WT-diabetic mice ($P = 0.017$; Figure 1F).

### 3.4 Generation of hyperglycaemic BMTx mice

For the in vivo component of this study, three groups of animals were generated: (i) non-diabetic mice reconstituted with eGFP$^+$ BMCs (non-diabetics); (ii) STZ-treated mice reconstituted with eGFP$^+$ BMCs (WT-diabetics); and (iii) STZ-treated mice reconstituted with eGFP$^+$/hGlo1$^+$ BMCs (GLO1-diabetics; Figure 2A). Hyperglycaemia was detected in 88% of STZ-treated mice, with an average of $20.8 \pm 1.1$ mmol, compared with $4.8 \pm 0.3$ mmol in non-diabetic mice.

![Figure 2](https://academic.oup.com/cardiovascres/article-abstract/101/2/306/325042)
(Figure 2B). At the end of the study, both groups of STZ-injected mice had significant weight loss compared with the non-diabetic control group (P ≤ 0.01; Figure 2C). GLO1 enzymatic activity was increased 1.4-fold in BMCs from GLO1-diabetic mice compared with those from WT-diabetic mice (P = 0.005; Figure 2D). Western blot of BMCs extracted from GLO1-diabetic mice showed successful reconstitution of the BM with cmyc-tagged eGFP/hGlo1 BMCs (Figure 2E).

3.5 GLO1 overexpression in BMCs restores ischaemia-induced CAC mobilization in diabetes

Prior to transplantation, the BMCs from the two donors (eGFP+ or eGFP+/hGlo1+) did not show any phenotypic differences (16.7 ± 1.2% FLK+, 2.0 ± 0.3% CXCR4+, 29.2 ± 0.9% c-kit+, and 58.3 ± 1.0% CD34+ cells). Following ligation, the mobilization of GFP+ CACs (CXCR4+, c-kit+, and VEGFR2+) was reduced in WT-diabetic mice compared with the non-diabetic group; whereas GFP+ CAC mobilization was maintained in GLO1-diabetic mice (Figure 3A). Specifically, during the early response post-ligation (Days 1–4), WT-diabetic mice had significantly less circulating GFP+ VEGFR2+ cells than the other two groups (P ≤ 0.048). Also, at later time points (Days 7 + 14), the number of circulating GFP+CXCR4+, GFP+VEGFR2+, and GFP+c-kit+ cells was not different between non-diabetic and GLO1-diabetic mice, but both were greater compared with the WT-diabetic group (P ≤ 0.05). Analysis of the early serum post-ligation (Days 1 + 4) revealed that both diabetic groups of mice (GLO-diabetic and WT-diabetic) had reduced levels of mobilizing and pro-angiogenic cytokines: SDF-1 was approximately two-fold higher, and VEGF was approximately three-fold higher in the non-diabetic group vs. the two diabetic groups (P ≤ 0.05; Figure 3B). At the later time points (Days 7 + 14), cytokine levels were not significantly different between the three groups (Figure 3B).

3.6 Neovascularization and perfusion is restored in GLO1-diabetic mice

Vascular density was assessed by α-SMA staining for arterioles and CD31 staining for capillaries in hindlimb tissue 2 weeks post-ischaemia (Figure 4A–H). The number of arterioles was greater in GLO1-diabetics (1.7 ± 0.3 arterioles/0.5 mm²) compared with WT-diabetic mice (0.5 ± 0.1; P = 0.01), but not different from non-diabetics (1.3 ± 0.3; P = 0.4; Figure 4G). Also, the number of CD31+ cells was greater in GLO1-diabetics (13.4 ± 2.0 ± 15 mm²) compared with WT-diabetic mice (8.75 ± 0.7; P = 0.038), but not different from non-diabetics (14.7 ± 0.5; P = 0.45; Figure 4H). Femoral artery ligation reduced perfusion in all groups (ischaemic/non-ischaemic hindlimb ratio of 0.5 ± 0.1; P < 0.001 compared with pre-op baseline; Figure 4I and J). In WT-diabetic animals, perfusion of the hindlimb distal to the ligation site showed no signs of recovery either at 7 or 14 days. At 7 days, blood flow in GLO1-diabetic mice (1.0 ± 0.2) was greater than in WT-diabetic mice (0.5 ± 0.1; P = 0.015). After 2 weeks, more blood flow was observed for GLO1-diabetic (1.1 ± 0.1) and non-diabetic (1.1 ± 0.2) mice compared with WT-diabetic mice (0.4 ± 0.0; P ≤ 0.012).

3.7 Gene expression of angiogenic factors in the ischaemic tissue

At Day 3 post-ligation, the transiently increased mRNA expression of Hif1-α and Vegfa in non-diabetic mice was greater compared with both diabetic groups (P ≤ 0.046), while eNos mRNA was not significantly different between the groups (Figure 5A). The level of eNos mRNA from the hindlimbs was significantly increased in WT-diabetic mice compared with non-diabetics at 2 weeks post-ischaemia (P ≤ 0.04; Figure 5B). A trend for increased expression of Vegfa mRNA was observed in the WT-diabetic group (vs. non-diabetic mice), while the expression of Hif1-α mRNA was higher in WT-diabetic mice than in the other two groups (P = 0.05; Figure 5B).

3.8 Cytokine and eNOS analysis in ischaemic tissue

The protein level of tumour necrosis factor-α (TNF-α), an inflammatory cytokine, was elevated in the hindlimb of WT-diabetic mice at 2 weeks post-ligation compared with the other two groups (P ≤ 0.0014; Figure 5C). Cytokine analysis of the tissue also revealed that VEGF content was significantly lower in WT-diabetic hindlimb muscle than in non-diabetic mice (P = 0.05; Figure 5C), while SDF-1 was reduced in WT-diabetic and GLO1-diabetic mice compared with non-diabetic controls (P ≤ 0.02; Figure 5C). Although the level of eNos mRNA was significantly increased in WT-diabetics at 2 weeks post-ligation, the protein detected was mainly in the monomeric form. The ratio of metabolically
Figure 4 Recovery of neovascularization and blood flow in GLO1-diabetic mice. (A–H) Representative images of α-SMA staining (A–C) for arterioles (red), and of CD31 staining (D–F) for capillaries (red); nuclei stained with DAPI (blue); scale bar = 50 μm. (G) The average number of blood vessels per 0.5 mm² (*P ≤ 0.02 for WT-diabetic vs. other groups; n = 5–6). (H) The average number of CD31⁺ cells per 0.15 mm² (*P ≤ 0.038 for WT-diabetic vs. other groups; n = 3). (I) Perfusion was measured by laser Doppler analysis, and data are presented as the average ratio of ischaemic to non-ischaemic limb blood flow (*P ≤ 0.0015 for GLO1-diabetic vs. WT-diabetic; †P = 0.012 for non-diabetic vs. WT-diabetic). (J) Representative images of perfusion over a period of 2 weeks post-ligation (red = highest perfusion; blue = lowest).
active eNOS dimer to non-functional eNOS monomer in WT-diabetic and GLO1-diabetic mice was significantly lower than in non-diabetic mice ($P \leq 0.05$; Figure 5D).
This suggests that BMCs overexpressing hGlo1 are more likely to respond to mobilization and homing signals generated by ischaemic tissue in the diabetic setting, which we did, in fact, observe in vivo. Early after ischaemia (1–4 days), serum levels of VEGF and SDF-1 pro-angiogenic cytokines in GLO1-diabetic and WT-diabetic mice were reduced compared with non-diabetics. Despite this, the mobilization of BM-CACs in GLO1-diabetic mice was equal to non-diabetic mice, whereas the numbers were reduced in the WT-diabetic group.

The viability of BMCs from GLO1-diabetic mice was greater than those from WT-diabetic mice when they were exposed to apoptosis-inducing conditions. Notably, GLO1-BMCs viability was equivalent to that of BMCs from non-diabetic mice. This may be explained by the higher level of Bcl-2 and Bcl-XL mRNA in GLO1-BMCs compared with WT-diabetic BMCs. Bcl-2 and Bcl-XL are anti-apoptotic factors, and MG has been shown to reduce Bcl-2 expression in retinal endothelial cells. Unlike BMCs from GLO1-diabetic
Hif-1α is elevated compared with the other two groups. Despite the increased B.V. and D.K. were supported by Canadian Graduate Scholarships from the Funding Acknowledgements Supplementary material.

It is noticed that the positive effects of BM reconstitution with hGlo1 overexpressing BMCs were obtained with a moderate 1.4-fold increase in GLO1 activity, measured in vitro. A consideration is that STZ administration may irreversibly damage some BMC subpopulations through DNA alkylation.28,29 While we cannot formally exclude that increased GLO1 activity through unknown mechanisms may lessen STZ-induced BMC cytotoxicity, it is more probable that the beneficial effects of hGlo1 over-expression in BMCs result from reduced hyperglycaemia-induced dicarboxyl stress. The reduction in oxidative stress (protein carbonyl levels) measured in GLO1-diabetic BMCs compared with WT-diabetic BMCs supports this.

High glucose in diabetes impairs HIF-1α expression leading to reduced expression of angiogenic factors.2,3 This was demonstrated early after induction of ischaemia (Day 3), where hindlimb expression of HIF-1α mRNA increased in non-diabetic mice, compared with the two diabetic groups. The low level of Hif-1α in the tissues of GLO1-diabetic and non-diabetic mice after 2 weeks likely reflects the restoration of blood flow and the absence of hypoxia. In contrast, ischaemia persisted in the WT-diabetic mice at Day 14, and Hif-1α mRNA was elevated compared with the other two groups. Despite the increased Hif-1α mRNA in WT-diabetic mice, this did not lead to elevated protein levels of HIF-1α-regulated cytokines SDF-1 and VEGF.

At 2 weeks, pro-inflammatory TNF-α expression was higher in the WT-diabetic group, suggesting the persistence of local inflammation, which was not observed in GLO1-diabetic mice. The level of eNos mRNA in WT-diabetic mice was also increased compared with the other two groups, probably driven by the lack of oxygen. The paradoxical increase of eNos expression during hypoxia in diabetic mice has previously been reported,1 arguing that chronic elevated expression of eNos mRNA does not result in production of functional eNOS dimer protein, which was also observed in this study.

In conclusion, our findings show that overexpression of GLO1 uniquely in the BM can protect BM-CACs’ viability and function, and is sufficient to overcome the defective neovascularization that is characteristic of diabetes. This may prove to be a stepping stone for developing strategies aimed at improving the efficacy of revascularization therapies in diabetic patients.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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